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Combination Protocols of Cytokine Therapy With Interleukin-3 and Granulocyte-Macrophage Colony-Stimulating Factor in a Primate Model of Radiation-Induced Marrow Aplasia

By Ann M. Farese, Douglas E. Williams, Fritz R. Seiler, and Thomas J. MacVittie

Single cytokine therapy with granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) has been shown to be effective in decreasing the respective periods of neutropenia and thrombocytopenia following radiation- or drug-induced marrow aplasia. The combined administration of IL-3 and GM-CSF in normal primates suggested that a sequential protocol of IL-3 followed by GM-CSF would be more effective than that of GM-CSF alone in producing neutrophils (PMN). We investigated the therapeutic efficacy of two combination protocols, the sequential and coadministration of recombinant human IL-3 and GM-CSF relative to respective single cytokine therapy, and delayed GM-CSF administration in sublethally irradiated rhesus monkeys. Monkeys irradiated with 450 cGy (mixed fission neutron:gamma radiation) received either IL-3, GM-CSF, human serum albumin (HSA), or IL-3 coadministered with GM-CSF for days 1 through 21 consecutively postexposure, or IL-3 or HSA for days 1 through 7 followed by GM-CSF for days 7 through 21. All cytokines and HSA were injected subcutaneously at a total dose of 25 μg/kg/d, divided twice daily. Complete blood counts (CBC) and platelet (PLT) counts were monitored over 60 days postirradiation. The respiratory burst activity of the PMN was assessed flow cytometrically, by measuring hydrogen peroxide (H_2O_2) production. Coadministration of IL-3 and GM-CSF reduced the average 16-day period of neutropenia and antibiotic support in the control animals to 6 days ($P = .006$). Similarly, the average 10-day period of severe thrombocytopenia, which necessitated PLT transfusion in the control animals, was reduced to 3 days when IL-3 and GM-CSF were coadministered ($P = .004$). The sequential administration of IL-3 followed by GM-CSF had no greater effect on PMN production than GM-CSF alone and was less effective than IL-3 alone in reducing thrombocytopenia. PMN function was enhanced in all cytokine-treated animals.

LINEAGE-SPECIFIC CYTOKINES, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte-CSF (G-CSF) have demonstrated therapeutic efficacy in reducing the periods of neutropenia associated with radiation- and drug-induced marrow aplasia.[1-3] Interleukin-3 (IL-3) has shown efficacy in stimulating the production of platelets (PLTs) in animals[4] and some patients, but is less effective in generating neutrophils (PMNs).[5,6]

Previous studies in normal primates have indicated that the combination of GM-CSF and IL-3 promoted a synergistic rise in peripheral white blood cells (WBC) and PLT levels when IL-3 was administered before GM-CSF.[7-9] Whereas the coadministration of GM-CSF and IL-3 resulted in diminished PMN production relative to GM-CSF alone (Farese et al, unpublished data). The sequential combination of IL-3 and GM-CSF is based on the concept that IL-3 will expand GM-CSF sensitive target cells for more efficient production of neutrophils in addition to generating an increase of PLT precursors. Whereas, the combined administration of IL-3 and GM-CSF, may result in downregulation of GM-CSF receptors by IL-3 and thus dampen the GM-CSF–induced increase in PMNs.

IL-3 regulates proliferation of early multipotential progenitor cells, as well as more committed progenitor cells of erythroid, granulocyte, and megakaryocyte lineages.[10] GM-CSF in addition to its lineage-specific effects, also possesses megakaryocyte colony-stimulating activity.[11-13] The in vitro activity of both IL-3 and GM-CSF has been shown to be additive with respect to stimulating larger colonies than either cytokine alone.[12-16]

Despite monotherapy with the CSFs, neutropenia and thrombocytopenia have remained as severe consequences following radiation or chemotherapy. We tested the therapeutic efficacy of combined IL-3 and GM-CSF protocols, both sequentially and coadministered, for the production of PMNs and PLTs in sublethally irradiated rhesus monkeys. We demonstrated that unlike previous studies in normal cynomolgus and rhesus monkeys,[7,8] coadministration of GM-CSF and IL-3 promoted accelerated PLT and PMN recovery in marrow-ablated rhesus monkeys relative to sequential cytokine therapy or treatment with either cytokine alone.

MATERIALS AND METHODS

Expression, Purification, and Analysis of Cytokines

Human GM-CSF and IL-3, were expressed in yeast and purified to homogeneity as previously described.[17] N-linked glycosylation sites were eliminated from the GM-CSF (Asn 27, Thr 39) and IL-3 (Asn 15, Asn 70) cDNAs by the oligonucleotide-directed site-specific mutagenesis.[18] Purified recombinant proteins were analyzed by amino acid compositional analysis, and N-terminal sequence analysis was performed by automated Edman degradation with an Applied Biosystems Model 477A protein sequencer (Applied Biosystems, Foster City, CA). Quantitation of the endotoxin content of purified recombinant proteins was performed with the *Limulus* as-

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Table 1. Neutropenia and Thrombocytopenia in Sublethally Irradiated and Cytokine-Treated Rhesus Monkeys: Duration and Mean Days of Cytopenia

	Neutropenia		Thrombocytopenia	
Treatment	Days	Duration	Days	Duration
HSA	16	(5-20 d)	10	(10-19 d)
GM-CSF	11*	(6-16 d)	8	(10-17 d)
IL-3	15	(6-20 d)	6*	(10-15 d)
IL-3 and GM-CSF (d 1-21)	6*†	(6-9, 12-13 d)	3*†	(11-13 d)
IL-3 (d 1-7), GM-CSF (d 7-21)	12	(6-17 d)	8	(10-17 d)
HSA (d 1-7), GM-CSF (d 7-21)	12	(6-17 d)	8	(10-17 d)

Monkeys, whole body irradiated with 450 cGy of mixed fission neutron gamma radiation were treated with control protein (HSA) or cytokines according to protocol. Neutropenia is an absolute neutrophil count of $<1,000 \times 10^2/\mu L$. Thrombocytopenia is a platelet count of $<30,000 \times 10^3/\mu L$.

* Significant statistical difference from HSA-treated controls.

† Significant statistical difference from sequential protocols and GM-CSF protocol.

say (Whittaker Bioproducts, Gaithersburg, MD) and was below the limits of detection for GM-CSF and IL-3.

Primate Studies

Animals. Domestic born male rhesus monkeys, *Macaca mulatta* (mean weight 2.9 ± 0.3 kg), were housed in individual stainless steel cages in conventional holding rooms of an American Association for Accreditation of Laboratory Animal Care accredited animal facility at the Armed Forces Radiology Research Institute (AFRRI). Monkeys were provided 10 air changes per hour of 100% fresh air, conditioned to 72°F ± 2°F with a relative humidity of 50% ± 20% and maintained on a 12-hour light/dark full spectrum light cycle, with no twilight. Monkeys were provided with commercial primate chow, supplemented with fresh fruit and tap water ad libitum. Research was conducted according to the principles enunciated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources, National Research Council.

Irradiation and clinical support. Monkeys placed in an aluminum restraining chair, following a prehabituation period, were total body irradiated (TBI) in a posterior-anterior direction using the AFRRI TRIGA reactor. They were exposed to a pulse (<50 milliseconds) of mixed (1:1, free in-air) fission neutron and gamma radiation to a total free in-air (skin surface dose) of 450 cGy. All exposures were monitored using ionization chambers, sulfur activation foils, and radioluminescent glass and silicon diodes. An antibiotic regimen was initiated prophylactically when the WBC was ≤1,000/μL and continued daily until the WBC was >1,000/μL for 3 consecutive days. Gentamicin (Lyphomed, Deerfield, IL) (1.5 mg/kg q12) and rocephin (Roche, Nutley, NJ) (100 mg/kg/d) were administered intramuscularly. Fresh, irradiated (1,500 cGy ^{60}Co) PLTs from a random donor pool (monkeys of >10 kg) were administered every other day when the PLT count was less than 30,000/μL.

Recombinant cytokine administration protocol. Beginning on day 1 after irradiation, monkeys were administered twice daily subcutaneous injections of either recombinant human IL-3 (25 μg/kg/d, n = 4), GM-CSF (25 μg/kg/d, n = 4), GM-CSF and IL-3 (25 μg/kg/d each, n = 4), or human serum albumin (HSA; 25 μg/kg/d, n = 5) as a control for 21 days. In studies of sequential growth factor administration, IL-3 (25 μg/kg/d) was administered for 7 days (beginning on day 1) followed by 14 days of GM-CSF (25 μg/kg/d, n = 4). Another cohort of animals received HSA for days 1 to 7, followed by 14 days of GM-CSF (25 μg/kg/d).

Peripheral Blood Analysis

Peripheral blood was sampled to assay complete blood (Model S Plus II, Coulter Electronics, Hialeah, FL) and differential counts (Wright-Giemsa Stain, Ames Automated Slide Stainer, Elkhart, IN). Baseline levels (BL) were obtained before irradiation.

Neutrophil activation studies. Heparinized peripheral blood was diluted with 0.83% NH_4Cl (10 minutes, 21°C) to lyse the red blood cells. The leukocytes were pelleted (400*g* for 10 minutes at 4°C), washed in Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (GIBCO, Grand Island, NY), resuspended in Dulbecco's phosphate-buffered saline (PBS; GIBCO) (5 to 10 × 10^6/mL), and maintained at 4°C. Cell viability was determined to be ≥95% by trypan blue exclusion.

Hydrogen peroxide (H_2O_2) production was measured as described by Bass et al.[19] The leukocytes (10^6 cells/mL) were incubated with 5 μmol/L 2', 7', dichlorofluorescin-diacetate (DCFH-DA; Kodak, Rochester, NY) in PBS for 10 minutes at 37°C. Cells were then placed on ice to inhibit spontaneous H_2O_2 production and assayed immediately. The fluorescence (FL) of the PMNs was measured flow cytometrically with a FACS analyzer interfaced to a Consort 30 computer system (Becton Dickinson, San Jose, CA) both before (control) and after (experimental) a 15-minute stimulation with phorbol-12-myristate, 13-acetate (PMA; Sigma, St Louis, MO) (100 ng/mL). Green FL was monitored between 515 and 545 nm after excitation by a mercury arc lamp with a 485/22 nm excitation filter. PMNs were differentiated by Coulter volume(s) and right angle light scatter properties. The percent change in H_2O_2 production was calculated as follows:

$$\frac{\text{Mean FL Intensity Experimental} - \text{Mean FL Intensity Control}}{\text{Mean FL Intensity Control}}$$

Results are reported as the percent change in mean fluorescence.

Statistical analysis. The Normal Scores Test was used to make pairwise comparisons of time. The test was performed using the software package StatXact (Cytel Software Corp, Cambridge, MA), and exact *P* values were obtained.

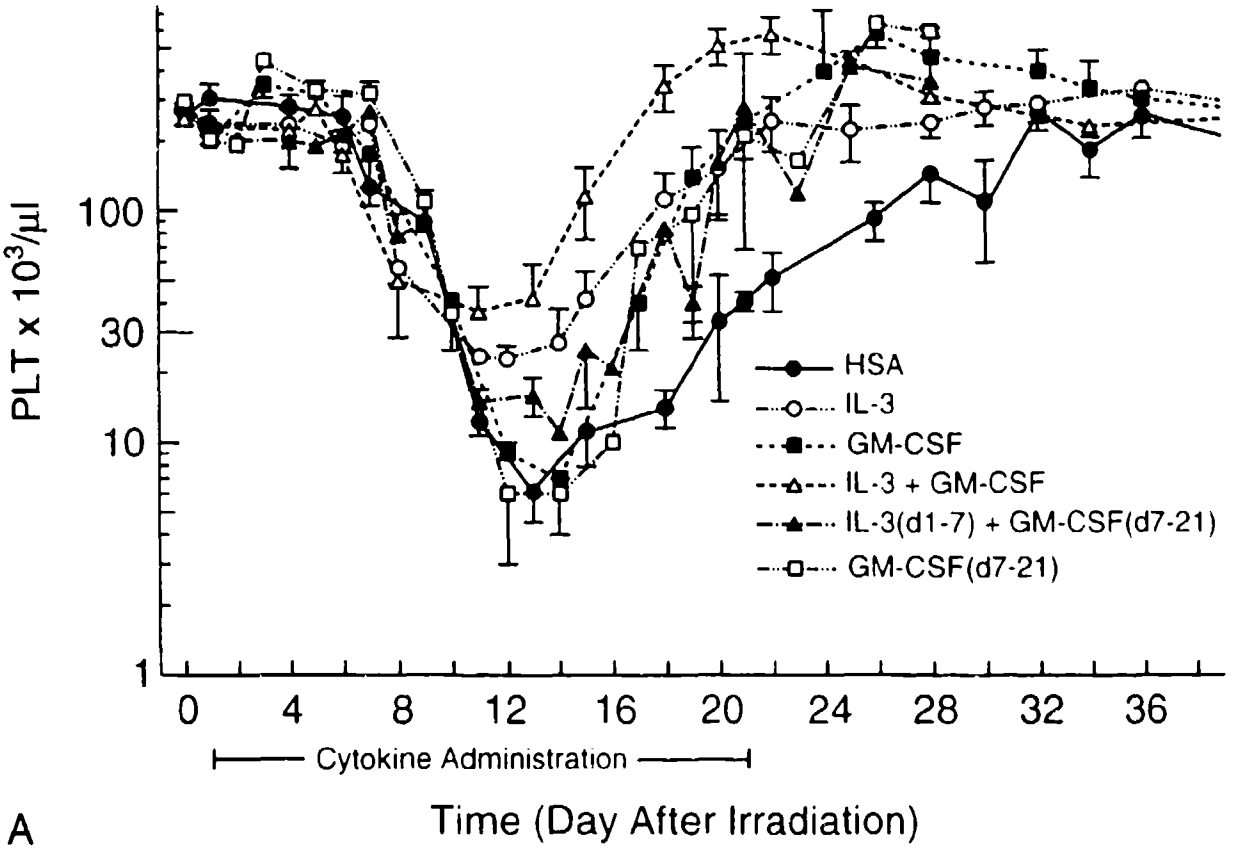
RESULTS

Effects of Cytokine Protocols in Sublethally Irradiated Rhesus Monkeys

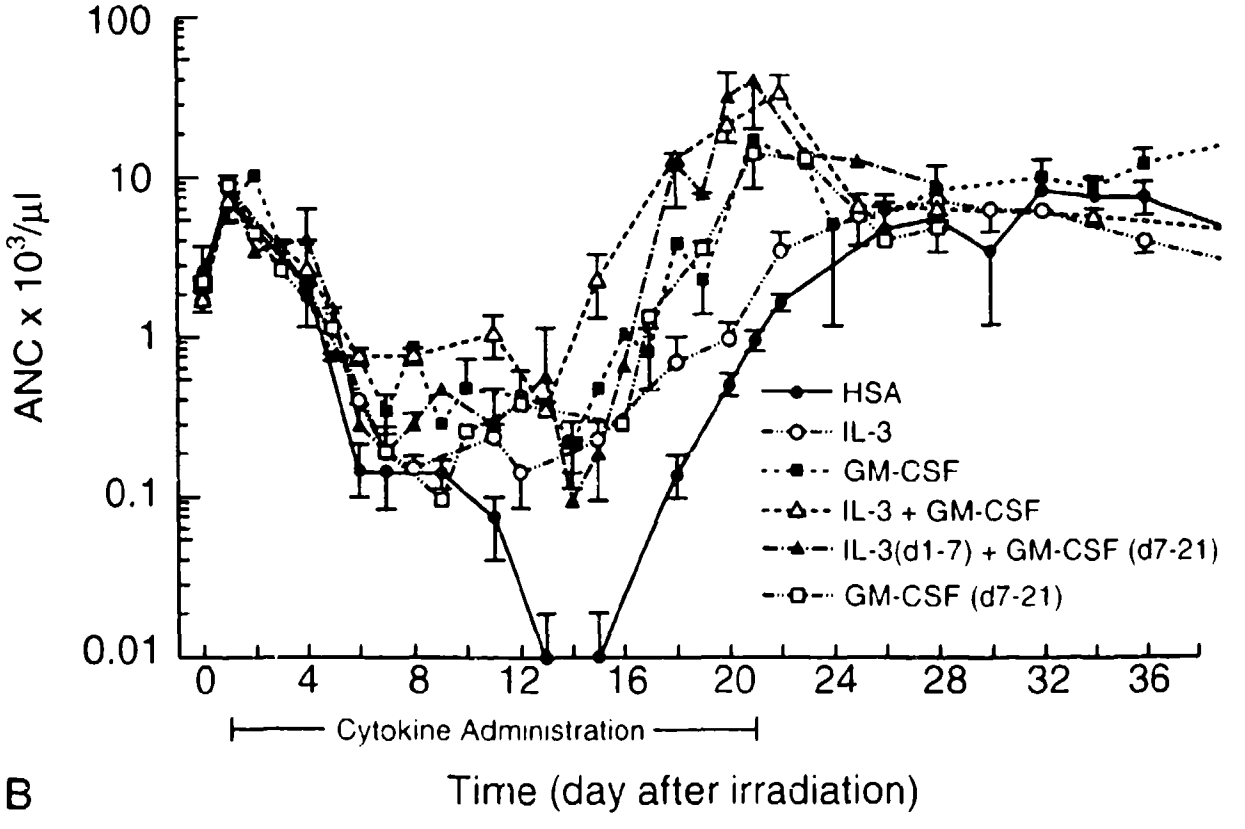
Exposure to 450 cGy TBI required both PLT transfusions and antibiotics to ensure 100% survival ($LD_{70/30}$ without clinical support, unpublished results). The HSA-treated control animals required antibiotic support for an average 16-day period (day 5 to 20) of neutropenia (ANC < 1,000/μL), and PLT support over a 10-day period (day 10 to 19) of severe thrombocytopenia (PLT < 30,000/μL) (Table 1 and Fig 1). Normal PMN and PLT levels were not attained until days 24 and 30, respectively (Fig 1).

Platelet Recovery

The administration of 25 μg/kg/d of GM-CSF shortened the duration of neutropenia in the irradiated primate. However, it did not significantly promote PLT recovery compared with HSA control animals, nor did it alter the depth


PLT x 10^3/µl
100
30
10
1
0
4
8
12
16
20
24
28
32
36
Cytokine Administration
HSA
IL-3
GM-CSF
IL-3 + GM-CSF
IL-3(d1-7) + GM-CSF(d7-21)
GM-CSF(d7-21)
Time (Day After Irradiation)

A


ANC x 10^3/µl
100
10
1
0.1
0.01
0
4
8
12
16
20
24
28
32
36
Cytokine Administration
HSA
IL-3
GM-CSF
IL-3 + GM-CSF
IL-3(d1-7) + GM-CSF (d7-21)
GM-CSF (d7-21)
Time (day after irradiation)

B

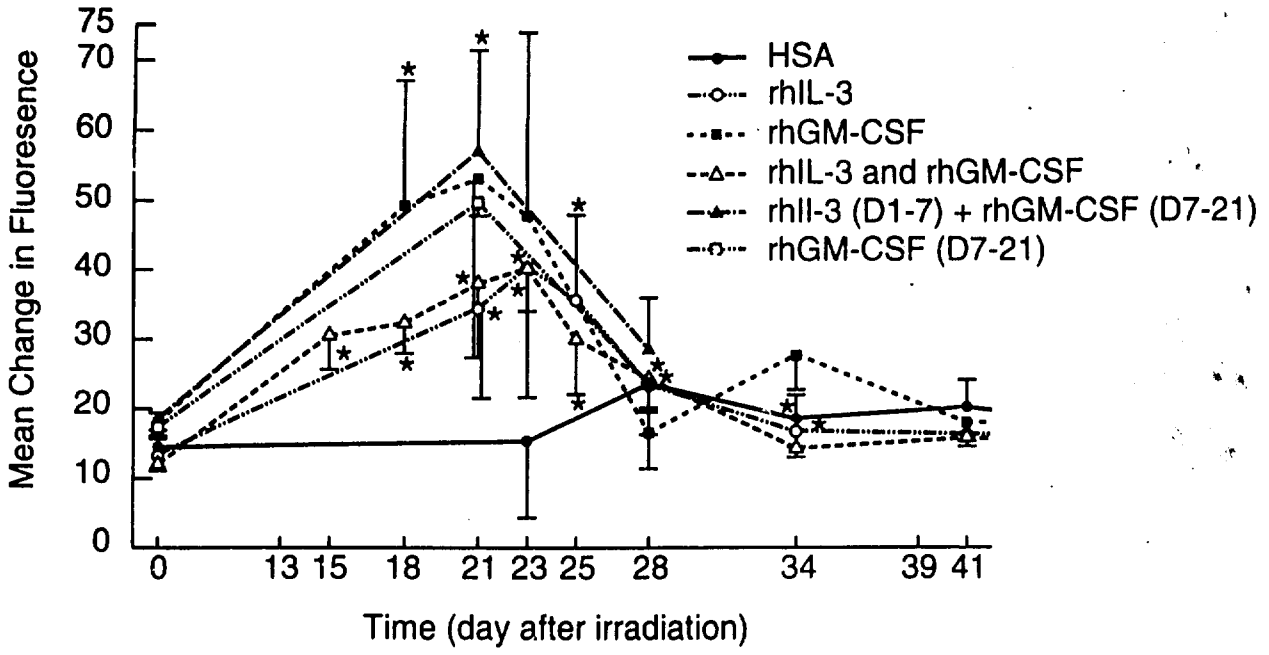


Fig 2. Hydrogen peroxide production as described in Methods by PMNs obtained from cytokine- or control-treated rhesus monkeys following sublethal irradiation. The mean values (± SEM) of the percent change in fluorescence/cell (10,000 PMNs/analysis) by each animal from its respective baseline are reported.

of the postirradiation nadir (Fig 1A). HSA- and GM-CSF-treated animals required from two to four PLT transfusions each during the nadir period. In contrast, IL-3 administration accelerated PLT recovery, with preirradiation levels attained by day 22 (Fig 1A). The PLT nadir in IL-3-treated animals as well as the duration of thrombocytopenia (day 10 to 15) was significantly different ($P = .005$) from HSA-treated controls (Fig 1A).

Coadministration of GM-CSF and IL-3 resulted in accelerated PLT recovery (Fig 1A), which reached preirradiation levels 5 days earlier than in IL-3–treated animals (day 17 v day 22). Animals coadministered IL-3 and GM-CSF were PLT transfusion-independent during the postirradiation nadir and although not statistically significant, further reduced the duration of thrombocytopenia 3 days versus IL-3–treated animals (Table 1). The duration of thrombocytopenia was significantly reduced by 7 days versus HSA-treated animals ($P = .004$) and 5 days versus the sequential IL-3/GM-CSF protocol ($P = .04$). The rate of PLT recovery, depth of the nadir period, and duration of thrombocytopenia in animals treated sequentially with IL-3 followed by GM-CSF did not differ significantly from that observed in animals receiving GM-CSF or IL-3 alone ($P = .19$) (Fig 1A). The PLT recovery in animals where GM-CSF administration began on day 7 postirradiation was similar to that seen when therapy was initiated on day 1 postirradiation (Fig 1A).

Neutrophil Recovery

The injection of either HSA, GM-CSF, IL-3, or GM-CSF and IL-3 beginning at day 1 postirradiation induced an early, rapid mobilization of PMNs into the systemic circulation (Fig 1B). PMN levels above normal were maintained for several days postirradiation, thereafter decreasing to their nadir. GM-CSF accelerated the recovery of circulating PMNs, and significantly ($P = .009$) reduced the duration of neutropenia by 5 days (day 6 to 16) compared with HSA-treated monkeys (day 5 to 20), and significantly reduced the depth of the nadir (Table 1 and Fig 1B). IL-3 treatment did not accelerate PMN recovery versus HSA-treated animals (Fig 1B and Table 1). Coadministration of GM-CSF and IL-3 elicited the most rapid return of PMN numbers to preirradiation levels (Fig 1B). An abortive increase of PMNs was noted at day 11 postexposure, which could not be sustained. A true PMN recovery followed several days hence. Antibiotic therapy was maintained through this increase, therefore antibiotic administration was reduced by 7 days in GM-CSF- and IL-3-treated animals. The duration of neutropenia was significantly reduced ($P = .006$) from 11 days to 6 days in animals that were coadministered GM-CSF and

Fig 1. Regeneration of the circulating platelet (A) and absolute neutrophil (B) counts (± SEM) following sublethal irradiation of rhesus monkeys treated with HSA or IL-3/GM-CSF cytokine protocols. Cytokines or HSA were administered over a 21-day protocol by twice daily subcutaneous injections as described in Methods.

IL-3 (Table 1) versus GM-CSF therapy alone. In addition, the coadministration of IL-3 and GM-CSF significantly (P = .01) reduced the duration of neutropenia (6 days) versus the sequential IL-3/GM-CSF protocol (12 days).

Sequential cytokine therapy provided no benefit in PMN production over GM-CSF alone (P = .19) or IL-3 alone (P = .19) (Fig 1B and Table 1). In addition, when GM-CSF administration was delayed until day 7, the animals exhibited an identical kinetic pattern of PMN recovery (P = .13) as those treated with GM-CSF for the entire 21 days (Fig 1B).

Neutrophil Function

PMNs produced during cytokine treatment with GM-CSF, IL-3, or GM-CSF and IL-3 were capable of producing significantly ($P \leq .05$) more H_2O_2 than normal, preirradiation PMNs (Fig 2). This priming effect decreased within several days after cessation of cytokine therapy. PMNs in HSA-treated animals were also primed for an increased respiratory burst in response to stimulation during the overshoot period after the nadir.

DISCUSSION

Severe neutropenia and/or thrombocytopenia remain as dose limiting sequelae following intensive cytotoxic therapy with drugs or radiation. The recombinant cytokine GM-CSF accelerates recovery of neutrophils[20-23] and maintains their functional capacity,[23] yet has little demonstrable effect on platelet recovery. In contrast, IL-3 promotes a slower increase in neutrophils and monocytes while accelerating the recovery of circulating platelet levels.[4-6] Recent in vitro[24,25] and in vivo[7-9,26] results of combined IL-3 and GM-CSF use suggest increased clinical efficacy of this cytokine combination.

The present studies were performed to test the in vivo efficacy of two combination regimens, either the coadministration or the sequential administration of IL-3 and GM-CSF, relative to their respective controls in a primate model of radiation-induced marrow aplasia. Surprisingly, the rate of neutrophil and platelet recovery was increased in response to coadministration of GM-CSF and IL-3 relative to the IL-3/GM-CSF sequential protocol. This would not have been predicted from studies in our laboratory, which showed a dampened response when these factors were coadministered to normal monkeys (Farese et al, unpublished data).

Stahl et al[9] recently evaluated the differential effects of coadministered, sequential, and single agent IL-3 and GM-CSF protocols on megakaryocyte maturation and platelet response in normal primates. They concluded that administration of IL-3 and GM-CSF in a sequential protocol was most efficient in stimulating thrombopoiesis by sequentially increasing megakaryocyte numbers and maturation. These cumulative effects were diminished by coadministration of the two cytokines. WBC were increased without altering the differential in all GM-CSF containing protocols. Sequential administration of IL-3 followed by GM-CSF had previously been shown to promote a synergistic stimulation of neutrophils and platelets in normal cynomolgus monkeys.[7,8]

However, sequential administration of IL-3 and GM-CSF in the present studies did not demonstrate a shortened recovery period, let alone synergy in the sublethally irradiated rhesus monkey. The kinetics of platelet recovery showed that the nadir and duration of thrombocytopenia (8 days) were not significantly different from the HSA, GM-CSF, or IL-3-treated controls. Relative to the GM-CSF monotherapy (11 days), the nadir and duration of neutropenia (12 days) were unchanged in animals treated with IL-3 followed by GM-CSF. However, once initiated, the production of neutrophils was rapid, reaching peak values greater than GM-CSF alone and equivalent to the coadministered IL-3/GM-CSF protocol. Recently Ganser et al[26] reported during a phase I study of tumor patients with normal hematopoietic capacity, that a sequential combination regimen of IL-3 and GM-CSF increased PMN counts but not PLT counts relative to IL-3 monotherapy. However, the PMN response to sequential IL-3/GM-CSF was not greater than that noted in the Herrmann et al study, when GM-CSF was administered alone.[27] In two additional clinical studies, sequential therapy with IL-3, followed by GM-CSF showed no benefit on myelopoiesis and thrombopoiesis versus monotherapy with GM-CSF[28] or IL-3.[29] Delay of GM-CSF therapy for 7 days without IL-3 priming, accelerated neutrophil recovery in a fashion similar to that observed in animals that received the full 21-day course of GM-CSF therapy. These results are comparable with those of Meisenberg et al,[30] who demonstrated that neutropenia due to a single dose of mechloroethamine can be equally reduced with both early and delayed initiation of G-CSF therapy. These data and those of Meisenberg et al[30] suggest that cytokine (G-CSF and GM-CSF) responsive target cells are available, at day 7 regardless of early GM-CSF or G-CSF administration. The responsive population requires several days to be generated. Early IL-3 administration alone does little for expanding the surviving GM-CSF target population in irradiated marrow.

These results are discordant with the conclusions drawn from in vivo results in normal primates, which showed that IL-3 expands progenitor cells that are responsive to subsequent GM-CSF administration. It has been recognized that the common functional characteristics of IL-3 and GM-CSF may in part be explained by shared binding capacities on hematopoietic cells. Both molecules compete for binding with equally high affinity for a subpopulation of cell surface receptors.[31-33] The competition between IL-3 and GM-CSF for receptor binding is explained by competition for the common β chain among the specific α subunit. In this case the β chain is rate limiting. It has been hypothesized by Lopez et al[34] that the α chain itself can deliver a growth signal by coupling directly to a signal transduction mechanism absent in mature cells. Progenitor cells may also have sufficient (nonlimiting) amounts of β chains allowing ligands to bind to and signal through the $\alpha\ \beta$ high-affinity receptor complex. Perhaps competition would not occur in this case. The irradiated, aplastic marrow may show a subpopulation of progenitor cells responsive to both IL-3 and GM-CSF, whereas target cells responsive to IL-3 alone are greatly reduced. It is worth noting that Stahl et al[11] showed

that megakaryocyte ploidy was significantly increased within 3 days in primates coadministered IL-3 and GM-CSF relative to the 7-day period required for either the sequential protocol or GM-CSF alone.

Our data also suggests increased presence and/or production of target cells responsive to the coadministration of IL-3 and GM-CSF and forecasts the potential efficacy of fusion proteins, such as the IL-3/GM-CSF protein PIXY321.[35,36] Data obtained using PIXY321 in this model, with respect to platelet and neutrophil production is comparable to that observed with coadministration of GM-CSF and IL-3.[37] An exception is the abortive rise in PMN production seen at day 11 with the coadministration of cytokines. This study suggests that coadministration of IL-3 and GM-CSF may provide a significant clinical advantage to existing cytokine therapies for myelosuppression. It also offers a cautionary note with regards to drawing conclusions about cytokine activity in myelosuppressed animals based on data obtained from normal animals.

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